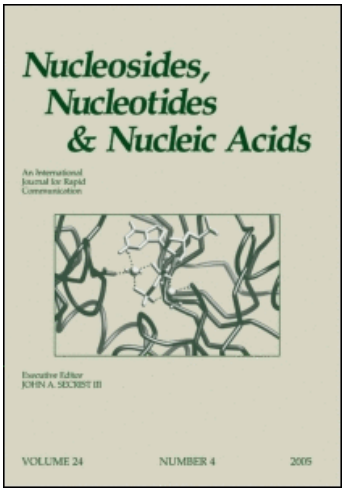

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article De Clercq, E. , Andrei, G. , Balzarini, J. , Hatse, S. , Liekens, S. , Naesens, L. , Neyts, J. and Snoeck, R.(1999) 'Antitumor Potential of Acyclic Nucleoside Phosphonates', Nucleosides, Nucleotides and Nucleic Acids, 18: 4, 759 — 771
To link to this Article: DOI: 10.1080/15257779908041563
URL: http://dx.doi.org/10.1080/15257779908041563

NUCLEOSIDES & NUCLEOTIDES, 18(4&5), 759–771 (1999)

ANTITUMOR POTENTIAL OF ACYCLIC NUCLEOSIDE PHOSPHONATES

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Acyclic nucleoside phosphonates such as HPMPC (cidofovir) and PMEA (adefovir) have been identified as broad-spectrum antiviral agents that are effective against herpes-, retro- and hepadnavirus infections (PMEA) and herpes-, pox-, adeno-, polyoma-, and papillomavirus infections (HPMPC). Here we show that HPMPC and PMEA also offer great potential as antitumor agents, through the induction of tumor cell differentiation (PMEA), inhibition of angiogenesis (HPMPC) and induction of apoptosis (HPMPC). *In vivo* tumor regressions have been noted for choriocarcinoma (PMEA) in rats, hemangioma (HPMPC) in rats and papillomatous lesions (HPMPC) in humans. Acyclic nucleoside phosphonates can be considered as a new dimension to the discipline of chemotherapy. They have a unique mode of action that is targeted at (viral or tumoral) DNA synthesis. They exhibit a pronounced and prolonged anti-viral and/or tumoral activity that can persist for days or weeks after a single administration. Most importantly, they have a uniquely broad spectrum of indications for clinical use, encompassing both DNA- and retrovirus infections, as well as various forms of cancer of both viral and non-viral origin.

1. Introduction

Acyclic nucleoside phosphonates (ANPs) (Fig. 1) can be considered as "mimics" or structural analogues of nucleotides. ANPs consist of an acyclic nucleoside moiety, i.e. acyclic cytidine or acyclic adenosine, to which a phosphonate group has been attached (through a P-C linkage, instead of the usual P-O-C linkage found in nucleotides). The prototype ANPs that have been constructed are cidofovir [HPMPC: (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine] and adefovir [PMEA: 9-(2-phosphonylmethoxyethyl)adenine]. They can be conceived as hybrid molecules between an acyclic nucleoside analogue (such as acyclovir) and a phosphonyl acid moiety (such as phosphonoformic acid), two molecules that are known to be targeted at viral DNA synthesis.

Whereas acyclic nucleoside analogues (such ad acyclovir) need three phosphorylation steps before they can interact, in their triphosphate form, with (viral or cellular) DNA synthesis, the acyclic nucleoside phosphonates need only two phosphorylations to be converted to their active metabolites, their diphosphate derivatives (Fig. 2). In this form they function as competitive inhibitors/alternate substrates in the DNA polymerase reaction, and, when incorporated, they act as DNA chain terminators and shut off DNA synthesis.

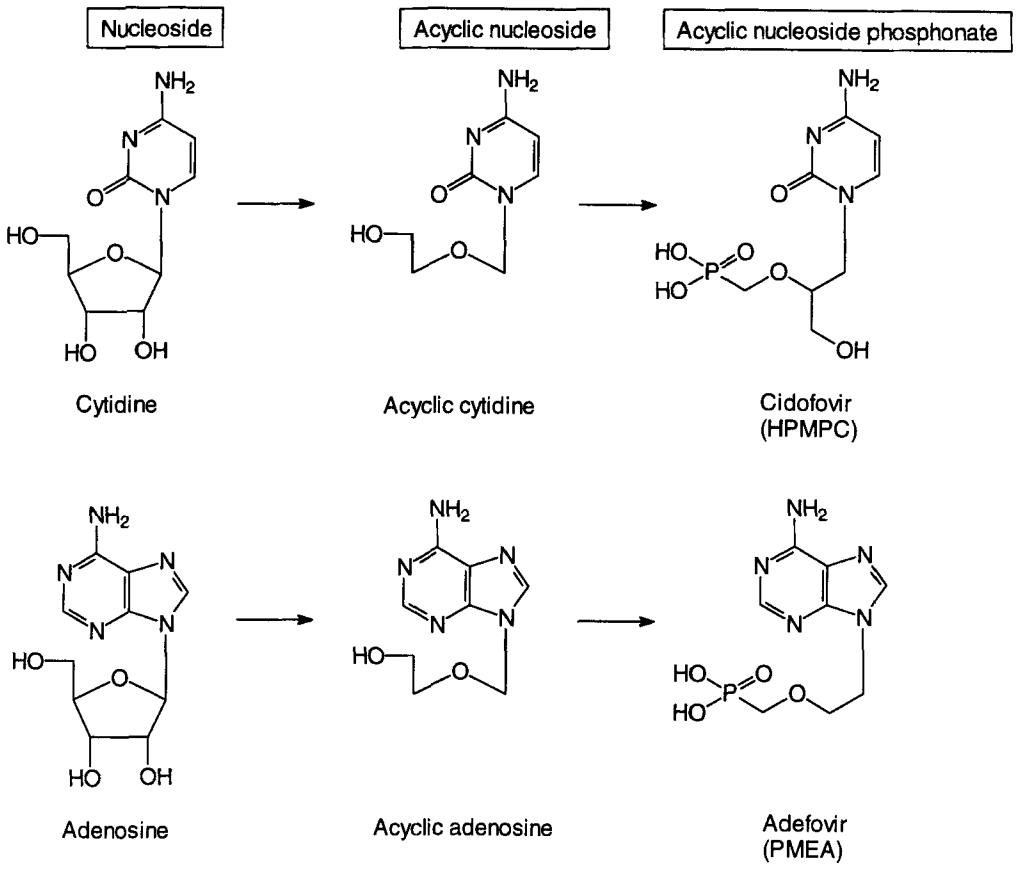

Fig. 1. Acyclic nucleoside phosphonates (ANPs): HPMPC and PMEA.

Akin to PMEA, HPMPC also needs two phosphorylations to be converted to its active form, HPMPCpp. As for PMEApp, the target of action of HPMPCpp is viral DNA synthesis. As has been shown particularly with cytomegaloviral DNA synthesis, two-consecutive incorporations of HPMPC are required before DNA synthesis is effectively shut off.

Acyclic nucleoside phosphonates have a remarkable antiviral activity spectrum: PMEA is active against herpesviruses [herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), cytomegalovirus (CMV), ...], hepadnaviruses [hepatitis B virus (HBV)] and retroviruses [human immunodeficiency virus (HIV), feline immunodeficiency virus (FIV), simian immunodeficiency virus (SIV), ...], whereas HPMPC is active against herpesviruses [those already mentioned for PMEA, and also human herpesvirus type 6 (HHV-6), type 7 (HHV-7) and type 8 (HHV-8)], adenoviruses, polyomaviruses [i.e. JC and BK, that are responsible for progressive multifocal leukoencephalopathy (PML)], papovaviruses [i.e. human

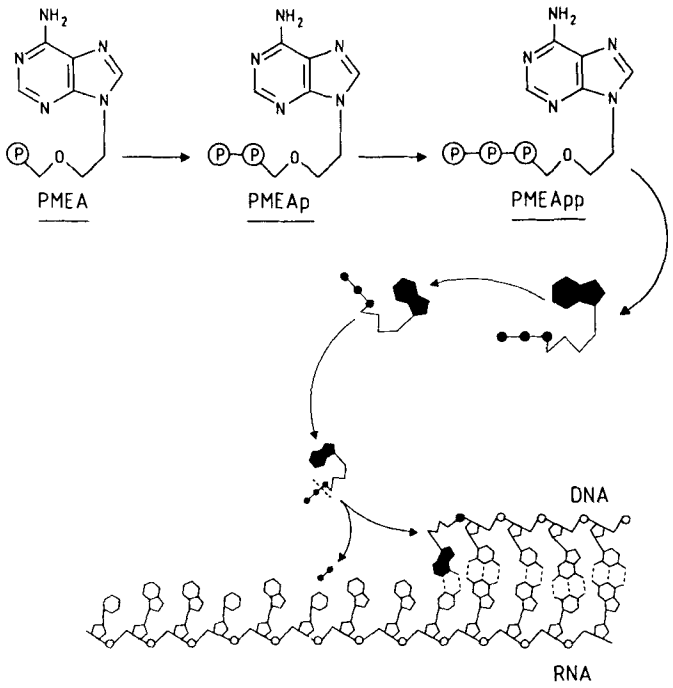

Fig. 2. Mechanism of action of acyclic nucleoside phosphonates as exemplified for PMEA.

papilloma virus (HPV)], and poxviruses [i.e. molluscum contagiosum virus (MCV)]. HPMPC (cidofovir, Vistide®) has been licensed (approved) for the treatment of CMV retinitis in immunosuppressed (i.e. AIDS) patients, and PMEA (adefovir), in its oral prodrug form [bis(POM)-PMEA (adefovir dipivoxil)], is currently in advanced (phase III) clinical trials for the treatment of HIV infections and has been made available for expanded access as Preveon™.

The antiviral properties of the acyclic nucleoside phosphonates were first described in 1986 and 1987[1,2] and have been the subject of several overviews, pertaining to the broad-spectrum antiviral potential of these compounds.[3-10] Here, we highlight the antitumor properties of the acyclic nucleoside phosphonates, a new dimension of their chemotherapeutic potential that was discovered during our recent investigations with these compounds.

2. PMEA is a strong inducer of differentiation of several tumor cell lines and shows marked in vivo antitumor activity in choriocarcinoma-bearing rats

PMEA is endowed with potent differentiation-inducing properties in several *in vitro* tumor cell lines, including human erythroleukemia K562, myeloid HL-60 and neuroblastoma SK-N-SH cells and rat choriocarcinoma RCHO cells.

In the presence of differentiation-inducing agents, K562 cells undergo changes similar to the normal maturation of red blood cells, such as the production of hemoglobin, the expression of glycophorin A on the cell membrane and increased activity of acetylcholine esterase. Benzidine staining of K562 cell cultures allows easy and rapid distinction between differentiated, hemoglobin-containing K562 cells which become blue-colored and undifferentiated cells which remain transparent.[11] Using this *in vitro* differentiation model, it was demonstrated that PMEA induces tumor cell differentiation in a time- and concentration-dependent manner. In K562 cell cultures that were continuously exposed to PMEA at 50 μM, initiation of differentiation became apparent at day 2, when 14% of the cells were benzidine-positive. The percentage of differentiated cells gradually increased in function of time until day 5, when 50% of the cells were differentiated.[12] Also, when K562 cells were exposed to 50 μM PMEA during different time periods (i.e., 1, 2, 3 or 4 days) and subsequently further incubated in drug-free medium until day 5, when differentiation was measured by benzidine staining, it was found that a 2- to 3-day exposure to PMEA is sufficient to afford at day 5 a differentiation level (39% to 51%) comparable to that obtained upon continuous exposure to 50 μM PMEA for 5 days (50%). Longer exposure times did not further raise the percentage of differentiated K562 cells. The fact that PMEA needs to be present for at least 2 days to be fully active may point to a metabolite, most likely diphosphorylated PMEA (PMEApp), as the active moiety, responsible for differentiation induction. Presumably, intracellular accumulation of PMEApp is required to achieve optimal differentiation. These data also indicate that it takes several days for the cells to establish the differentiated phenotype. The dose dependence of PMEA-induced K562 cell differentiation was demonstrated by incubating K562 cells with varying concentrations of PMEA (i.e., 0, 50, 100, 250 or 500 μM) for 24 hours. The percentage of benzidine-positive cells was recorded 3, 5, 7 and 10 days after drug removal and transfer of the drug-treated cells to PMEA-free growth medium. Irrespective of the time at which differentiation was measured, the percentage of hemoglobin-containing K562 cells gradually increased with higher drug doses.[12] Apparently, higher extracellular PMEA concentrations yield higher intracellular levels of the active PMEA metabolite(s) and thus, more pronounced biological effects. Accordingly, we found that PMEA uptake and phosphorylation is not saturated in K562 cells at an extracellular PMEA concentration as high as 2.5 mM. This experiment also revealed the long-lasting differentiation-inducing activity of PMEA : the differentiation reached its maximum level at day 5 after drug removal (following a 24-hour exposure), but the effect of the 24-hour PMEA exposure was still pronounced at 10 days after its removal.[12] Such long-term biological activity of PMEA may at least in part be attributed to the long intracellular half-life of the active form PMEApp.

The rat choriocarcinoma RCHO cell line represents another tumor cell model that was used to study the differentiation-inducing properties of PMEA. The RCHO cell line consists of rapidly

proliferating cytotrophoblast cells, which can differentiate into hormonally active syncytiotrophoblasts called 'giant cells'.[13] The morphological changes accompanying differentiation of RCHO cells can easily be observed microscopically. Secretion of progesterone and induction of alkaline phosphatase activity are additional, easily measurable differentiation markers.[14] Moreover, a monoclonal antibody, 22H6, that reacts specifically with an antigen present on cytotrophoblasts but not giant cells has been developed. Another monoclonal antibody, 28G4, reacts with both cell types. Hence, the ratio of 22H6 to 28G4 binding reflects the differentiation stage of the RCHO cells in the cell culture.[14] The effect of PMEA was evaluated on the different biochemical parameters of the *in vitro* choriocarcinoma differentiation model. The alkaline phosphatase activity gradually increased from 100% (untreated control) to 491% for PMEA concentrations varying from 0 to 50 μM.[14,15] Progesterone production showed a similar dose-response effect compared to a background level of 0.39 μg extracellular progesterone/100 ml culture medium of untreated control cells, progesterone concentrations of 0.47, 0.92 and 2.61 μg/100 ml (i.e., 120%, 236% and 669% of control) were measured in the extracellular medium of RCHO cell cultures exposed to PMEA at 10, 20 and 50 μM, respectively.[14,15] In addition, gradually increasing PMEA concentrations (from 0 to 50 μM) caused a gradual decrease in the 22H6 *versus* 28G4 antibody binding ratios (from 1.3 to 0.5), reflecting a shift of the RCHO cell population from predominantly cytotrophoblasts to mainly giant cells.[14] The *in vitro* differentiation-inducing potential of PMEA was found to be equivalent to that of methotrexate (MTX), which is currently the drug of choice for the treatment of choriocarcinoma in humans.[14,15]

When grafted under the kidney capsule of syngeneic WKA/H rats, RCHO cells give rise to extremely aggressive, hemorrhagic tumors.[16] Hence, this rat choriocarcinoma model may be useful to investigate the *in vivo* differentiation-inducing properties of drugs. We have found that PMEA, like MTX, is a potent inhibitor of the growth of the highly aggressive choriocarcinoma tumors in the rat model.[15,16] In untreated animals, massive invasive RCHO tumors, covering the whole surface of the kidney and resulting in a marked enlargement of the kidney, were observed at day 10 after tumor cell grafting. Daily treatment with PMEA at 25 mg/kg/day (intraperitoneally), initiated on the day of tumor cell grafting, afforded a marked reduction in tumor size: i.e., smaller tumors and slight, if any, enlargement of the kidney at day 10. Increasing the PMEA dose to 50, 100 or 250 mg/kg/day resulted in a gradual increase of the antitumor effect of the compound. At the highest dose tested, i.e. 250 mg/kg/day, PMEA completely suppressed tumor growth.[15,16] The antitumor activity of PMEA persisted for at least 10 days after termination of drug treatment. The residual small foci of tumor tissue that were still present after PMEA treatment (100 mg/kg/day during 10 days) did not expand but further regressed during the 10-day drug-free follow-up period.[16] In addition, delayed treatment with PMEA at a dose of 200 mg/kg/day, started at a time point where choriocarcinoma tumors of moderate size had already developed (i.e., 5 days after tumor cell inoculation), arrested further tumor growth and even induced regression of the tumors.[16]

In conclusion, PMEA proved to be a strong inducer of tumor cell differentiation in human erythroleukemia K562 and rat choriocarcinoma RCHO cells, and exhibited a pronounced antitumor activity against rat choriocarcinoma *in vivo*.

3. HPMPC confers potent inhibition of polyomavirus-induced hemangioma formation in rats, presumably due to its inhibitory effect on angiogenesis

Hemangiomas are angiomatous disorders characterized by the proliferation of capillary endothelium with accumulation of mast cells, fibroblasts and macrophages and represent the most frequent tumors of infancy.[17,18] They appear shortly after birth in 10% of all newborns and continue to grow very rapidly for the following 6 months. This proliferative phase is generally followed by regression of the tumor during the next 6-10 years. However, a small percentage of these tumors do not follow this course. Instead, they may develop into life-threatening hemangiomas by obstructing vital organs or cause hemorrhage, anemia, infections or Kasabach-Meritt syndrome, which may be fatal if left untreated. With the possible exception of interferon,[19-21] there is at present no effective treatment for these complex juvenile hemangiomas .

We have found that intraperitoneal infection of 4-day-old rats with $10^{9.7}$ PFU of murine polyomavirus (mPyV) results in the development of cerebral hemangiomas [which appear macroscopically at 7 days post infection (p.i.)], followed by the appearance of cutaneous and intramuscular hemangiomas at 14 days p.i. Rapid growth of the cerebral hemangiomas, associated with severe hemorrhage and anemia results in death of the rats within 3 weeks p.i.[22]

Proliferating human hemangiomas are vWF-positive [von Willebrand factor (a marker for endothelial cells)] and express high levels of PCNA [PCNA: proliferating cell nuclear antigen (a marker for proliferating cells)].[23] In order to investigate the relevance of the rat model for the pathology of human hemangiomas, we carried out an immunohistochemical analysis on hemangiomas in brains dissected at different times p.i. At 4 days p.i., lesions consisting of immature (vWF-negative), PCNA-positive endothelial cells were detectable. From day 4 to day 11 after infection, endothelial cells evolved into lumenlike structures containing erythrocytes. These cells showed a very clear positive signal for PCNA and numerous mitotic figures. Multiple hemangiomas of various size were present at day 11; in some of these hemangiomas the endothelial cells expressed vWF, indicating the presence of mature hemangiomas at this time point. From days 14 to 21, hemangiomas were composed of multiple cystic cavities with septation, others had converged into a single, huge blood-filled cyst. They expressed consistently both PCNA and vWF.

When treatment with HPMPC (25 mg/kg, subcutaneously, once a week) was initiated at 3, 6 or 9 days p.i., a complete suppression of development of intramuscular and cutaneous lesions was observed.[24] Furthermore, all animals in which treatment was initiated at 3 days p.i. survived, and 25-

40% survival was noted when treatment was started at 6 or 9 days p.i. Treatment was continued for 6 weeks. Survivors were kept for 6 months at which time autopsy (and histological examination) revealed that the animals were tumor-free. A marked delay in tumor-associated mortality was observed for those animals in the day 6 and day 9 group that ultimately succumbed [i.e. mean day of death (MDD): 23.0 ± 3.8 for the control group as compared to 37.7 ± 4.4 ($p<0.0005$) and 28.0 ± 1.0 ($p<0.005$) for the day 6 group and day 9 group, respectively].

Even at a dose as low as 5 mg/kg, once a week, subcutaneous treatment with HPMPC led to a marked protection (i.e. delay in mortality). When the start of treatment was delayed until 9 days p.i., at which time cerebral hemangiomas were already clearly visible (as determined by dissection of a parallel control group), a weekly dose of 5 mg/kg of HPMPC, still caused a marked delay in tumor-associated mortality (MDD: 23.2 ± 1.8, as compared to 18.5 ± 0.6 for the control group, $p<0.05$). Although hemangiomas were observed in the brains of those animals that succumbed following HPMPC therapy, their number was remarkably lower and, in contrast to the controls, almost no hemorrhage was noted in the brains of the treated animals.

When 4-day-old rats were infected with a 10-fold lower virus input than in the above experiments,[24] cerebral hemangiomas developed within 14 days p.i. and cutaneous or intramuscular lesions did not develop. Treatment with HPMPC (25 mg/kg, subcutaneously, once a week) was started at different times after infection and all animals were anesthetized at 4 weeks p.i. Their organs (brains, lungs, kidneys, spleen and liver) were examined macroscopically and histologically for the appearance of hemangiomas. Macroscopically detectable cerebral hemangiomas were observed in all control (untreated) animals. No hemangiomas or other tumors were detected in any of the other organs. HPMPC therapy, started before the appearance of brain tumors, inhibited the formation of hemangiomas: i.e. 0% and 40% of the rats developed cerebral hemangiomas when treatment was started at respectively 3 or 9 days p.i., as compared to 100% in the control group. Furthermore, the hemangiomas of the control group were clearly visible after dissection, whereas the hemangiomas in the day 9 treatment group could only be visualized after histological examination. In addition, HPMPC caused stabilization of already established hemangiomas. Indeed, HPMPC treatment, initiated at 14 days p.i. (at a time when brain lesions were detectable in a parallel control group), resulted in tumors that at 4 weeks p.i. were significantly smaller in size than control tumors.

To explain the efficacy of HPMPC in the mPyV-induced hemangioma rat model, we wanted to assure whether mPyV continued to replicate in rats or not. To this end we first performed a titration for infectious virus in tissue homogenates from brains (and other organs) dissected at different times p.i. No infectious virus could be detected in any of these organs. Second, a dot blot DNA-DNA hybridization assay was performed using target DNA extracted from the brains of both control (untreated) and HPMPC-treated animals (dissected at different times p.i.). No DNA

corresponding to VP1 (i.e. viral capsid protein, which is produced during a lytic virus infection) could be detected in any of these brains. In contrast, VP1 DNA could be readily detected in 1 ng of total cellular DNA extracted from mPyV-infected MO cell cultures. Therefore, the amount of viral DNA in 25 mg of brain tissue must well be < 1 ng, indicating that there is no virus replication going on. Third, a semi-quantitative PCR for VP1 was performed on brain, lungs, liver, spleen and kidney, collected at different times after infection with mPyV and several dilutions of the virus stock were used as standards. The amount of VP1 DNA decreased with time after infection and, with exception of the brain, where a weak signal was still detectable 18 days p.i., no signal was obtained in the other organs collected at 18 days p.i. As in the untreated animals, a comparable decrease in VP1 DNA over time was detected in the organs of those rats that had been treated with HPMPC: i.e., at 18 days p.i. only a weak signal was observed in spleen and lungs.

In conclusion, no virus replication could be detected in the mPyV-infected rats, indicating that transformation of the endothelial cells by the viral middle T protein may be sufficient to induce the formation of hemangiomas. Since (i) HPMPC was found to be active against established cerebral hemangiomas, thus after transformation of the endothelial cells had occurred, an antitumor (i.e., anti-angiogenic) action, rather than inhibition of viral replication, may account for the marked efficacy of HPMPC in this hemangioma model. HPMPC should be further explored for its potential in the treatment of vascular tumors and, in particular, life-threatening juvenile hemangiomas.

4. HPMPC effects a complete regression of papillomatous tumors in patients, which may, at least in part, be mediated by the induction of apoptosis

Of the acyclic nucleoside phosphonate derivatives, 9-(2-phosphonomethoxyethyl)guanine was the first shown to be a potent inhibitor of the growth of papillomavirus-induced tumors in the cottontail rabbit model.[25] Later, HPMPC was shown to be a potent inhibitor in the same model.[26]

HPMPC has also been shown to be effective in the treatment of different clinical presentations of HPV-induced epithelial cell proliferation. Of the patients presenting with anogenital lesions, the first to be treated and reported[27] were three AIDS patients with severe, relapsing anogenital HPV lesions. HPV type 16 was identified in the lesions of the three patients and they were all treated with daily applications of cidofovir cream or gel containing 1% HPMPC. Two of them showed a response after a few days, characterized by the disappearance of the proliferating cells leaving the place to diffuse ulceration and later to normal mucosa. The third patient was treated for five weeks before a complete response could be witnessed.

A phase I/II double blind, placebo-controlled study on the safety and efficacy of HPMPC topical gel for the treatment of patients with HPV-associated anogenital warts (condylomata acuminata), demonstrated the potential of HPMPC in the treatment of such lesions.[28] Thirty patients

were enrolled in the study, 19 in the cidofovir gel 1% and 11 in the placebo. The proportion of patients with complete response was 47% for the cidofovir-treated *versus* 0% for the placebo ($p = 0.006$); while, when complete and partial responses were pooled, the numbers were 84% for the cidofovir-treated *versus* 18% for the placebo ($p < 0.001$). There was an equal proportion of application site reactions (pain, pruritus, ulceration and rash). No patient had evidence of systemic toxicity during drug administration. Only 1 of 8 patients with complete response had a recurrence at the initial lesion site, at 120 days after cessation of drug therapy, while that group of patients had a median follow-up of 210 days.

We have previously reported the results of a study with local intratumoral injections of HPMPC in an immunocompetent 69-year old woman with hypopharyngeal/esophageal papillomatous lesions, that were PCR-positive for HPV types 16 and 18, that relapsed after extensive surgery and that also failed to respond to Nd-YaG laser photocoagulation and alpha-interferon treatment.[29] HPMPC was given at 1.25 mg/kg, with a sclerosing needle, through the biopsy channel of a video-endoscope directly into the tumor. The first four injections were given at an interval of one week at the level of the hypopharynx. The next three injections were given at an interval of 3 to 5 weeks in both the hypopharynx and the esophagus. An important decrease in the volume of the hypopharyngeal tumor was already observed after the second injection of HPMPC. Upon the subsequent injections, the lesions became progressively smaller and then completely disappeared. Concomitantly, the dysphagia disappeared as well. A later recurrence localized at another site of the esophagus was successfully treated by three additional HPMPC injections. Now, 5 years after the last injection, the patient is still disease-free. At the given dosage (1.25 mg/kg/week) HPMPC was well tolerated and no hematotoxicity, nephrotoxicity, hepatotoxicity, gastrointestinal toxicity or any other toxic side effects could be detected. Furthermore, locally, there were no ulceration, scarring or fibrosis. Three additional patients with a similar pathology (esophageal papillomatous lesions) have been treated since the first case was published. All three showed a remarkable regression of their tumor upon local injection of HPMPC (our unpublished data).

Respiratory papillomatosis is a rare and often severe disease, usually localized in the larynx. It may cause respiratory distress and even life-threatening obstruction of the airways. Treatment is usually based on the evaporation of the lesions with a CO_2 laser, but microsurgery, cytotoxic and/or cytostatic drugs, interferon and vaccines are also used. We reported the efficacy of HPMPC in 17 patients with severe respiratory papillomatosis.[30] HPMPC at a concentration of 2.5 mg/ml was injected directly in the different laryngeal papillomatous lesions during microlaryngoscopy under general anaesthesia. Complete disappearance of the lesions was observed in 14 patients. Of the three remaining patients, one progressed while under treatment of HPMPC after an initial marked response. One patient had a partial remission and remained stable for more than one year after the

last injection and finally one patient was lost of follow-up after the fourth injection. No inflammation, scarring or fibrosis were observed at any of the sites where HPMPC was injected. In addition, no systemic toxicity was noted.

Cervical intraepithelial neoplasia (CIN) is a proliferative process with differential severity depending on both the extension of the proliferation in the epithelium and the presence of cellular atypia. HPV has been clearly associated with such lesions, particularly those HPV types that are qualified as high risks. Cidofovir 1% in gel was applied three times, every other day, on the cervix of each of 15 women with biopsy-proven CIN grade III. Within one month after the start of treatment, the cervix was surgically removed. For 7 of the 15 patients, the histology showed a complete response, while 5 patients had a partial response characterized by the persistence of CIN I, II or III lesions in the depth of the glands, 1 patient had a dysplasia of lower grade (CIN I) and 2 patients did not show difference in the histology. The complete response was confirmed by PCR negativation in 4 of the 7 patients. Cidofovir was not toxic to the normal epithelium and systemic toxicity was not observed.[31,32]

We have evaluated the *in vitro* cytostatic effects of HPMPC and other acyclic nucleoside phosphonate derivatives on HPV-positive cells, compared to primary human keratinocytes (PHK). In PHK cells, isolated from normal human cervix, there was no significant decrease in the 50% cytostatic concentration (CC_{50}) in function of time for the different acyclic nucleoside phosphonates tested, while for the different HPV-positive cell lines [CK1 (HPV-33-positive), CaSki (HPV-16-positive), SiHa (HPV-16-positive) and HeLa (HPV-18-positive)] the treatment with the different phosphonates resulted in an inhibition of cell proliferation that increased in function of time. This effect was particularly striking for HPMPC. Thus, the CC_{50} values for HPMPC decreased 34- to 75-fold from 26 to 143 μg/ml at day 3 to 0.7 to 2.0 μg/ml at day 7. This marked antiproliferative effect in function of time was much less pronounced in the case of PMEA.[33]

To study the process leading to cell death following treatment of the cells with HPMPC, different approaches were used, based on the changes in several cellular processes that occur during apoptosis: (i) induction of CPP32 (caspase-3) protease activity, (ii) translocation of phosphatidylserine (PS) from the inner part of the plasma membrane to the outer layer, (iii) release of nuclear matrix protein (NMP) in a soluble form, (iv) cellular DNA fragmentation, (v) increase in the percentage of cells in apoptotic phase following cell cycle analysis.[34,35]

By using a specific ELISA, we could demonstrate a direct relationship between the amount of soluble NMP and the number of dead cells for those HPV-containing cells that had been treated with HPMPC. By studying the membrane phospholipid changes, necrotic cells could not be detected among these cells treated with HPMPC. In order to demonstrate the activation of proteases, the CPP32 (caspase-3) activity was measured. HPMPC afforded a marked increase in the protease

activity, compared to the level observed in the untreated cells. Also, the DNA fragmentation assay showed a dose-dependent amount of fragmented BrdU-labeled DNA following HPMPC treatment, again arguing for the induction of apoptosis. Finally, the cell cycle analysis by flow cytometry of HPV-positive cells treated with HPMPC confirmed the induction of apoptotic cell death. Treatment of CK-1 cells with HPMPC resulted in a decrease in the percentage of cells in the G0/G1 phase and an accumulation of cells in the S phase.

In conclusion, HPMPC has demonstrated a great potential in the treatment of severe HPV-induced proliferative lesions, either laryngeal, esophageal/hypopharyngeal or genital. HPMPC has also been shown to induce apoptosis, as monitored by a variety of parameters, in a time- and dose-dependent manner in a number of HPV-positive cell lines. It is tempting to attribute the regression of papillomatous tumors, as observed with HPMPC in patients, to the induction of apoptosis.

5. Conclusion

The acyclic nucleoside phosphonates cidofovir [(*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine, HPMPC] and adefovir [9-(2-phosphonylmethoxyethyl)-adenine, PMEA] have been identified as broad-spectrum antiviral agents. PMEA is effective against herpes-, retro- and hepadnavirus infections, and HPMPC is effective against herpes-, pox-, adeno-, polyoma- and papillomavirus infections. Here we show that HPMPC and PMEA also offer great potential as antitumor agents, through the induction of tumor cell differentiation (PMEA), inhibition of angiogenesis (HPMPC) and induction of apoptosis (HPMPC).

PMEA proved to be a potent inducer of differentiation of various tumor cells, including human erythroleukemia K562 cells, rat choriocarcinoma RCHO cells and human acute promyelocytic leukemic HL-60 cells. *In vivo*, PMEA was able to achieve a dose-dependent suppression of the growth of highly aggressive choriocarcinoma tumors in rats.

HPMPC was found to completely suppress the development of cutaneous, peritoneal and cerebral hemangiomas that are associated with polyomavirus (PyV) infection in rats. The formation of hemangiomas depended on transformation of the endothelial cells by PyV and not on viral replication (as shown by dot blot DNA hybridization, semiquantitative PCR and titration for infectious virus). The suppressive effect obtained with HPMPC treatment, started at a time at which the hemangiomas, were fully established could therefore be ascribed to an antitumor, i.e. anti-angiogenic, action.

HPMPC has also been found to cause a complete and permanent regression of human papilloma virus (HPV)-induced tumors in patients, i.e. squamous papilloma of the hypopharynx/esophagus, anogenital papillomatous lesions (condylomata acuminata), cervical intraepithelial neoplasia (CIN) grade III and laryngeal papillomatous lesions. The mechanism by

which HPMPC effected the complete regression of papillomatous tumors in patients may well be based on apoptosis (programmed cell death), since HPMPC was shown to induce apoptosis (as monitored by a variety of parameters, including DNA fragmentation) in a time- and dose-dependent fashion in a number of HPV-containing cell lines.

REFERENCES

1. De Clercq, E.; Holý, A.; Rosenberg, I.; Sakuma, T.,-, Balzarini, J.; Maudgal, P.C. *Nature*, **1986**, *323*, 464-467.
2. De Clercq, E.; Sakuma, T.; Baba, M.; Pauwels, R.; Balzarini, J.; Rosenberg, I.; Holý, A. *Antiviral Res.*, **1987**, *8*, 261-272.
3. De Clercq, E. *Biochem. Pharmacol.*, **1991**, *42*, 963-972.
4. De Clercq, E. *Rev. Med. Virol.*, **1993**, *3*, 85-96.
5. Naesens, L.; Balzarini, J.; & De Clercq, E. *Rev. Med. Virol.*, **1994**, *4*, 147-159.
6. De Clercq, E. *Verh. K. Acad. Geneesk. Belg.*, **1996**, *58*, 19-49.
7. Naesens, L.; Snoeck, R.; Andrei, G.; Balzarini, J.; Neyts, J.; De Clercq, E. *Antiviral Chem. Chemother.*, **1997**, *8*, 1-23.
8. De Clercq, E. *Clin. Microbiol. Rev.*, **1997**, *10*, 674-693.
9. De Clercq, E. *Intervirology*, **1997**, *40*, 295-303.
10. De Clercq, E. *Collect. Czech. Chem. Commun.*, **1998**, *63*, 480-506.
11. Balzarini, J.; Verstuyf, A.; Hatse, S.; Goebels, J.; Sobis, H.; Vandeputte, M.; De Clercq, E. *Int. J. Cancer*, **1995**, *61*, 130-137.
12. Hatse, S.; Balzarini, J.; De Clercq, E. *Nucleosides Nucleotides*, **1995**, *14*, 649-652.
13. Verstuyf, A.; Sobis, H.; Goebels, J.; Fonteyn, E.; Cassiman, J.J.; Vandeputte, M. *Int. J. Cancer*, **1990**, *45*, 752-756.
14. Hatse, S.; Naesens, L.; De Clercq, E.; Balzarini, J. *Biochem. Pharmacol.*, **1998**, in press.
15. Hatse, S.; Naesens, L.; Degrève, B.; Vandeputte, M.; Waer, M.; De Clercq, E.; Balzarini, J. *In*: Purine and Pyrimidine Metabolism in Man IX, edited by Griesmacher et al., Plenum Press, New York, **1998**, pp. 605-609.
16. Hatse, S.; Naesens, L.; Degrève, B.; Segers, C.; Vandeputte, M.; Waer, M.; De Clercq, E.; Balzarini, J. *Int. J. Cancer*, **1998**, *76*, 595-600.
17. Fishman, S. J.; Mulliken, J. B. *Pediatric Surgery*, **1993**, *40*, 1177-1200.
18. Mulliken, J. B.; Young, A. E. Vascular Birthmarks, Hemangiomas and Malformations. Philadelphia, PA, Saunders, **1988**.
19. Ezekowitz, A. B.; Mulliken, J. B.; Folkman, J. *N. Engl. J. Med.*, **1992**, *326*, 1456-1463.
20. White, C. W.; Sondheimer, H. M.; Crouch, E. C.; Wilson, H.; Fan, L. L. *N. Engl. J. Med.*, **1989**, *320*, 1197-1200
21. Orchard, P. J.; Smith, C. M.; Woods, W. G.; Day, D. L.; Dehner, L. P.; Shapiro, R. *Lancet*, **1989**, *2*, 565-567.
22. Liekens, S.; Verbeken, E.; Vandeputte, M.; De Clercq, E.; Neyts, J. Submitted for publication, **1998**.
23. Takahashi, K.; Mulliken, J.B.: Kozakewich, H.P.W.; Rogers, R.A.; Folkman, J.; Ezekowitz, R.A.B. *J. Clin. Invest.*, **1994**, *93*, 2357-2364.
24. Liekens, S.; Andrei, G.; Vandeputte, M.; De Clercq, E.; Neyts, J. *Cancer Res.*, **1998**, *58*, 2562-2567.
25. Kreider, J.W.; Balogh, K.; Olson, R.O.; Martin, J.C. *Antiviral Res.*, **1990**, *14*, 51-58.
26. Kurtzman, G.; Pickel, M.; Christensen, N.; Kreider, J. 33rd Interscience Conference on

Antimicrobial Agents and Chemotherapy, New Orleans, USA, 17-20 October **1993**. Abstract no. 1593.

27. Snoeck, R.; Van Ranst, M.; Andrei, G.; De Clercq, E.; De Wit, S.; Poncin, M.; Clumeck, N. *N. Engl. J. Med.*, **1995**, *333*, 943-944.;
28. Snoeck, R.; Bossens, M.; Parent, D.; Delaere, B.; De Greef, H.; De Clercq, E.; Safrin, S.; McGuire, B.; Jaffe, H.S. Eleventh International Conference on Antiviral Research, San Diego, California, USA, 5-10 April 1998. *Antiviral Res.*, **1998**, *37*, no 27, A47.
29. Van Cutsem, E.; Snoeck, R.; Van Ranst, M.; Fiten, P.; Opdenakker, G.; Geboes, K.; Janssens, J.; Rutgeerts, P.; Vantrappen, G.; De Clercq, E. *J. Med. Virol.*, **1995**, *45*, 230-235.
30. Snoeck, R.; Wellens, W.; Desloovere, C.; Van Ranst, M.; Naesens, L.; De Clercq, E.; Feenstra, L. *J. Med. Virol.*, **1998**, *54*, 219-225.
31. Snoeck, R.; Arens, M.; Muller, C.; Noel, J.C.; De Clercq, E.; Bossens, M. Tenth International Conference on Antiviral Research, Atlanta, Georgia, USA, 6-11 April 1997. *Antiviral Res.*, **1997**, *34*, no 108, A72.
32. Snoeck, R.; Noel, J.C.; Muller, C.; De Clercq, E.; Bossens, M. *Lancet*, **1998**, submitted for publication.
33. Andrei, G.; Snoeck, R.; Piette, J.; De Clercq, E. 16th International Papillomavirus Conference, Siena, Italy, 5-11 September **1997**. Abstracts, p. 237.
34. Andrei, G.; Snoeck, R.; De Clercq, E. Eleventh International Conference on Antiviral Research, San Diego, California, USA, 5-10 April 1998. *Antiviral Res.*, **1998**, *37*, no 26, A47.
35. Andrei, G.; Snoeck, R.; Schols, D.; De Clercq, E. The 1998 Molecular Biology of Small DNA Tumor Viruses Meeting, Wisconsin, Madison, USA, 14-19 July **1998**. Abstracts, p. 175.